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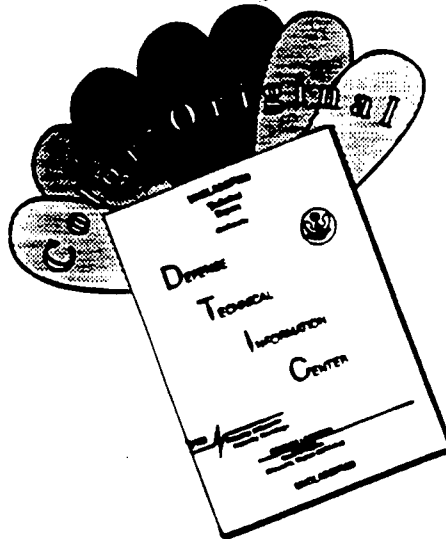   Fort Detrick, Frederick, Maryland   21702-5012

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The expression levels of a total of 15 cell cycle regulatory proteins have been determined in a panel of breast cancer and normal breast epithelial cell lines, as well as in a number of breast tissue and normal breast epithelial tissue samples. The results of these analyses indicate the presence of a defect in the expression of cyclin D1, Rb and/or the cyclin dependent kinase inhibitor protein, p16, in essentially all the breast cancer cell lines and tissues studied. The degree of overexpression of cyclin D1 is most closely reflected by changes in mRNA levels, although gene amplification and in one case an increase in half-life of the protein also contribute. Homozygous deletion of the p16 gene has been found to be a frequent mechanism for the absence of this tumor suppressor protein in breast cancer. Construction of replication incompetent adenovirus vectors for high efficiency transfection of breast cancer cells with genes encoding antisense cyclin D1 and sense p16 has been essentially completed. Human breast cancer cell lines tumorigenic in nude mice have been identified and will be transfected with these adenoviral vectors to directly test and confirm the role of cyclin D1 and p16 expression in breast cell tumorigenicity.

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Introduction:

In the first year of the grant we established in the laboratory a panel of available breast cancer and normal breast epithelial cell lines, and characterized them with respect to the expression of a number of cell cycle regulatory proteins, including cyclin D1 and Rb (retinoblastoma) protein, as well as three additional proteins whose importance in cell cycle regulation became clear after the original grant was written. In addition, we examined 4 breast tumor tissues and 1 unpaired normal breast epithelial tissue sample for the expression of the same group of proteins.

In the second year of the grant, due in part to the rapidly growing number of cell cycle regulatory components known to interact with cyclin D1 and Rb, we have determined the expression level of 7 additional proteins in this panel of breast cancer and normal breast cell lines. We also repeated and refined the analysis of cyclin D1 and Rb, correcting several false negative and false positive observations which were due to protease activity and antibody cross reactivity. In addition, we characterized the expression of cyclin D1 and Rb in 4 matched pairs of breast cancer tissue and normal breast epithelial tissue from the same patients. The results of these analyses indicate the presence of a defect in the expression of cyclin D1, Rb and/or the recently discovered cyclin dependent kinase inhibitor protein, p16, in essentially all the breast cancer cell lines and tissues studied.

Although not specifically proposed in the original application, we have extended our studies of cyclin D1 and p16 to include a determination of the mechanisms responsible for the overexpression or deletion of these proteins in many breast cancer cell lines, since this information is important for understanding the fundamental defects in breast cancer cells that contribute to malignancy.

We have also initiated a major effort towards construction of antisense cyclin D1 vectors, in order to test the effects of cyclin D1 ablation on the transformed properties of breast cancer cell lines (Specific Aim 3). This effort, which we had hoped to initiate towards the end of the first year, was delayed because of the need to expand the number of cell cycle regulatory proteins screened for, in the light of new developments in understanding cell cycle regulation. We originally proposed to construct plasmid vectors encoding antisense cyclin D1 and isolate stable breast cancer cell transfectants for study of cell growth characteristics. However, developments in the use of replication-incompetent adenovirus vectors, which allow essentially 100% transfection of cell populations within less than 24 hours, provided an opportunity for much more powerful technology to test the role of cyclin D1 and other cell cycle regulatory proteins in breast cell transformation. After overcoming an unexpectedly large number of technical problems, we have successfully constructed plasmid vectors encoding antisense cyclin D1 and are currently cotransfecting these with adenovirus vectors in order to isolate recombinant adenovirus encoding antisense cyclin D1. Since it is anticipated that different viral recombinants will express different levels of antisense transcript, it will probably not be necessary to use constructs containing promoters of different strengths, as originally proposed for plasmid vectors. Although not part of the original specific aims, we have also cloned a sense orientation gene for p16 into a plasmid vector and will begin isolation of a recombinant adenoviral vector expressing this protein

shortly. Using the same procedures, we also plan to construct recombinant adenovirus expressing sense and antisense Rb in year three or four (Specific Aim 4). These viral vectors should allow extensive investigation of the contributions of defects in cell cycle regulatory proteins to the malignant state in breast cancer cells. In anticipation of the availability of these viral reagents, we have tested and demonstrated the tumorigenicity in nude mice of a breast cancer cell line overexpressing cyclin D1, as well as a control breast cancer cell line in which cyclin D1 is not expressed, in order to provide an experimental system in which effects of cell cycle regulatory protein expression can be studied.

Finally, we have initiated a study of potential correlations between mutations in the breast cancer tumor suppressor genes BRCA1 and BRCA2, and specific defects in cell cycle regulatory machinery. While well outside the original specific aims, we feel it is essential to incorporate seminal new findings into our overall strategy for exploring the relationship between cell cycle regulation and breast cancer. These studies are being carried out on our panel of breast cancer cell lines as well as on a panel of six additional matched pairs of breast cancer tissue and normal breast epithelial tissue. The indication from preliminary results that loss of functional BRCA1 and/or BRCA2 may be associated specifically with tumors that retain Rb expression will be pursued in the coming year, in hopes of gaining more information about the defects that contribute to breast cell malignancy.

Because of the additional work undertaken with respect to newly recognized cell cycle regulatory proteins and the development of viral vectors, study of the serum (growth factor) dependence of cyclin D1 expression, originally proposed for year 1, and the analysis of cyclin-cdk complexes, originally proposed to take place throughout the 4 year period, have been delayed and will be carried out in years 3 and 4.

Materials and Methods

Breast cancer cell lines and tumor material

A panel of 12 breast cancer cell lines were obtained both from the American Type Culture Collection (DU4475, MDA-MB-157, MDA-MB-175VII, MDA-MB-361, MDA-MB-415 and Hs578T), and the University of Colorado Tissue Culture Core Facility (COLO 591, MCF-7, MDA-MB-231, MDA-MB-330, T-47D and ZR75.1). One normal, immortalized, non-transformed breast epithelial cell line, MCF-12A, and one normal breast epithelium cell line transformed with SV40 T antigen (TAg), HBL-100, were also obtained from the University of Colorado Tissue Culture Core Facility. The cell lines COLO 591, COLO 742 and DU4475 were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 18 mM HEPES, 1% non-essential amino acids, 1 mM sodium pyruvate and 2 mM L-glutamine. MCF-12A was cultured in Ham's F12/DME (1:1) supplemented with 10% fetal calf serum, 20 ng/ml EGF, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin and 10 μg/ml insulin. Hs578T was cultured in DMEM media supplemented with 10% fetal calf serum, 0.45% glucose, 0.4% sodium bicarbonate and 2 mM L-glutamine. The remaining cell lines were cultured in MEM media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 10 mM HEPES, 1% non-essential amino acids, 2 mM L-glutamine and 6 ng/ml insulin. Four matched pairs of frozen normal and tumor tissues were obtained from breast cancer patients who had undergone mastectomy by Dr. Wilbur Franklin, Dept. of Pathology, University of Colorado Medical School.

Antibodies

The following primary antibodies were obtained from Upstate Biotechnology, Lake Placid, NY: anti-cyclin A, anti-cyclin B1, anti-cyclin D1, anti-cdk2, anti-cdk4, anti-cdk6, and anti-PSTAIRE kinase. Anti-cyclin D2, anti-cyclin D3, anti-cyclin E and anti-p16 were obtained from Pharmingen, and anti-p21 and anti-p27 were obtained from Santa Cruz Biotechnology Inc. Anti-pRb and PCNA were gifts from Dr. Wen-Hua Lee (University of Texas Health Science Center, San Antonio, TX) and Dr. Wilbur Franklin (University of Colorado Health Sciences Center, Denver, CO) respectively. The horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad.

DNA probes and oligonucleotides

A 1.1 kb cyclin D1 cDNA probe, pPl-8, was obtained from Invitrogen. The B-actin probe was a gift from Dr. Robert Gemmill (Department of Medicine, University of Colorado Health Sciences Center).The c5.1 (p16) oligonucleotide sequences were obtained from Kamb et al., (1994) and those corresponding to the D9S199 locus were obtained from the GDB. The PACCMVpLpA plasmid was a gift from Dr. Jerome Schaack (Department of Microbiology, University of Colorado Health Sciences Center).

Growth of adenovirus (327$_{Bst}$B-gal) DNA

The 327$_{Bst}$B-gal vector was a gift from Dr. Jerome Schaack (Department of Microbiology, University of Colorado Health Sciences Center). The vector was engineered to replace the E1 region with the E.coli B-galactoside gene under the control of the human cytomegalovirus promoter, and thus lacks replicative and packaging functions (Schaack et al., 1995). Propagation of the vector requires the human kidney embryonal cell line, 293

that expresses the E1 genes. Cultures of 293 cells were grown in DMEM supplemented with 10% fetal calf serum, 0.45% glucose, 0.4% sodium bicarbonate and 2 mM L-glutamine. A high titer stock of 327_{Bst}B-gal was added to a 70-80% confluent 6 cm plate of 293 cells at a multiplicity of infection of at least 25. The cells were incubated at 37°C for 24-48 hours until they exhibited a cytopathic effect, at which time the medium was neutralized and the cells pelleted. The virus was released by alternative freezing and thawing in liquid nitrogen four times and the crude viral supernatant stored at -70°C. To isolate pure DNA, the viral supernatant was overlayed on a CsCl step gradient and centrifuged for one hour at 36,000 rpm. The viral band was removed and subjected to CsCl gradient centrifugation overnight at 24,000 rpm. Following collection of the virus band by side puncture, it was diluted with one volume of water and ethanol precipitated.

Staining of cells with X-5-bromo-4-chloro-3-indolyl B-D-galactoside (X-gal)

Monolayers of cells were rinsed with cold PBS and fixed for 5 minutes with cold 2% paraformaldehyde and 0.2% glutaraldehyde in PBS, followed by a second rinse with PBS. Cells were then incubated in 1 mg/ml X-gal in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS at 37°C for 14-18 hours.

Protein extraction and western blot analysis

Cells were harvested from each of two 175 cm^2 flasks at 50-70% confluence. Following two washes in PBS, approximately 2 X 10^6 cells were removed for fluorescence-activated cell sorter (FACS) analysis by the University of Colorado Cancer Center Flow Cytometry Core to determine cell cycle distribution. The remaining cells were resuspended in Laemmli sample buffer (Laemmli., 1970), boiled for 4 minutes, sheared through a 26-gauge syringe needle, aliquoted, and stored at -80°C. Individual pieces of tumor and normal tissues each weighing approximately 0.2 g, were crushed to a fine powder under liquid nitrogen, lysed in Laemmli sample buffer and processed as described above for the cell lines.

Approximately 50 µg of each protein extract were subjected to SDS/PAGE (Laemmli., 1970) and transferred either to nitrocellulose (Schleicher and Schuell) or Immobilon P (Amersham) membranes for 45 minutes at 0.45 A using the Genie Electrophoretic Blotter (Idea Scientific, Minneapolis). Membranes were stained with Ponceau dye to control for equal loading and immunodetection performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

DNA isolation and Southern blot analysis

DNA was prepared by incubating cells or finely minced solid tissues at 55°C in lysis buffer (10 mM Tris pH 8.0, 2.0 mM EDTA pH 8.0, 10 mM NaCl, 5% SDS) containing 1 mg/ml Proteinase K. The samples were then subjected to two phenol-chloroform extractions and one chloroform:isoamylalcohol (24:1) extraction, followed by ethanol precipitation.

Cell line DNA (8 µg) was digested with EcoRI, size-fractionated on 1% agarose gels, transferred onto nylon filters, and hybridized to ^{32}P randomly labeled probes (Feinberg and Vogelstein, 1983). The hybridization conditions have been described elsewhere (Ibson et al., 1987). Autoradiography of the membranes was performed at -70°C for 1-3 days using Amersham Hyperfilm-MP.

DNA analysis by "touchdown" polymerase chain reaction

A 20 μl reaction mixture overlaid with a drop of mineral oil contained a final concentration of 200 ng of genomic DNA, PCR buffer supplied by Promega (10 mM tris-HCL, pH 8.3; 5 mM KCl; 0.1% gelatin); 200 μM dNTP's; 1.5 mM MgCl$_2$; and 0.06 units μl^{-1} Taq polymerase. For amplification of a single locus, 0.2 μM of either the C5.1 (p16) primers or D9S199 primers were used, and for amplification of both loci by multiplex PCR, 0.2 μM of the C5.1 (p16) primers and 0.25 μM of the D9S199 primers were used. Following an initial denaturation period of 7 minutes at 95°C, the DNA was subjected to 20 cycles of amplification consisting of denaturation for 1 minute at 94°C, annealing for 1 minute, with a starting temperature of 55°C and decreasing by 0.5°C per cycle for the first 20 cycles, and elongation for 1 minute at 72°C. The final 10 cycles consisted of denaturation for 1 minute at 94°C, annealing for 1 minute at 45°C and elongation for 1 minute at 72°C followed by a final elongation step of 10 minutes at 72°C. The PCR products were subjected to electrophoresis on a 3% nusieve gel, stained with ethidium bromide and visualized with ultraviolet (uv) light.

PCR analysis using radio-labeled oligonucleotides.

The detection of dinucleotide repeat polymorphic loci involved the use of one unlabeled and one end-labeled primer in the PCR reaction mixture. The end-labeling reaction contained 1 μg of primer, 40 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 5 mM DTT, 15 μCi γ[^{32}P]ATP, and eight units of T4 polynucleotide kinase. Following incubation of the reaction mixture at 37°C for 30 minutes, the primer was diluted with water and added to the PCR reaction together with the unlabeled primer, each at a concentration of 0.1 μM. The resulting PCR products were electrophoresed on a 7% acrylamide/32% formamide gel, dried and exposed to X-ray film for up to 16 hours.

RNA isolation and northern blot analysis

RNA was isolated from log phase cultures of the cell lines and subjected to northern blot analysis as described by Hensel et al. (1990). The hybridization conditions were the same as those described above in the Southern blot analysis section.

Results:

Expression of cell cycle regulatory proteins in breast cancer.

We expanded the panel of normal and tumor breast epithelial cell lines and tissues described in the previous progress report to include 4 matched pairs of tumor and normal breast tissues obtained from breast cancer patients at surgery by Dr. Wilbur Franklin (Department of Pathology, University of Colorado Health Sciences Center). Normal breast tissue samples were obtained at a site removed from the tumor lesion (Table 1). Furthermore, the number of antibodies to cell cycle regulatory proteins used in the analysis of the cell lines was increased from eight to fifteen. The additions include antibodies to PCNA, the cyclins A, B1, D2, D3, and the inhibitors p21^{CIP1} and p27^{KIP1}. One of the breast cancer cell lines, BT-483, has been removed from our panel of cell lines owing to difficulties in culturing sufficient numbers of cells for a complete analysis of protein expression using our large panel of antibodies.

The cell lines were initially immunoblotted with anti-PCNA, the processivity subunit of DNA polymerase δ, which like the Human cdc2 Kinase (PSTAIRE), appeared to be expressed at relatively constant levels in the cell line panel. We also observed relatively constant levels of the S-phase and M-phase specific cyclin's, A and B1, respectively. Small differences in the expression of these cyclins between cell lines reflected variation in cell cycle distribution as identified by FACS analysis. Whereas cyclins D2 and D3 were expressed at barely detectable levels in the cell line panel, cyclin E protein was expressed at a relatively constant level in all cell lines (as described in the first progress report) with particularly elevated expression in DU4475 and MDA-MB-157. Interestingly, we observed marked variation in the expression both of p21 and p27 proteins ranging from a complete lack of expression to over-expression of the proteins relative to the normal breast cancer cell line, MCF-12A, in the breast cancer cell lines (Figure 1). However, there did not appear to be a correlation between the expression of either of these two proteins and any of the other cell cycle proteins assessed. Future functional studies have been planned to help elucidate the significance of the variation in expression of p21 and p27.

Combined over-expression of cyclin D1 and inactivation of p16 frequently overcome the Rb-mediated G1/S-block in breast cancer.

The previous progress report described a correlation between Rb and cyclin D1 expression in all but three of the breast cancer cell lines analyzed, COLO 591, ZR75.1 and MDA-MB-175VII. Since then, we have repeated the analysis of the panel of cell lines for the expression of both Rb and cyclin D1 using three independently isolated protein extracts per cell line. Significantly, both ZR75.1 and MDA-MB-175VII, which initially appeared to lack Rb expression, demonstrated normal levels of Rb protein expression in the newly isolated extracts. Further analysis of the original extracts from these two cell lines confirmed the indication that the Rb protein had been degraded by the action of proteases thus giving rise to falsely negative results. Consistent with the original extract, the three new COLO 591 extracts showed a lack of Rb protein expression but virtually undetectable levels of cyclin D1 compared to the moderate levels described in the original report. The signal detected initially using the cyclin D1 antibody has since been identified in three independent western blots to correspond to the homologous cyclin D3 protein (Figure 1).

Taking into account these revised findings, we are now able to state that that in all 12 of the breast cancer cell lines analyzed (and the normal breast epithelial cell lines, MCF-12A and HBL-100), there is a perfect correlation between Rb and cyclin D1 protein expression. Nine of the twelve tumor cell lines showed concomitant expression of Rb and cyclin D1 proteins with six of the nine expressing abnormally elevated levels of cyclin D1

protein relative to that shown by MCF-12A. The degree of over-expression ranged from approximately 4-5-fold (MDA-MB-175VII and MDA-MB-361) to 10-fold (MCF-7, MDA-MB-330, ZR75.1 and MDA-MB-415) as quantitated by densitometric analysis and normalization to PCNA signal. Three of the twelve tumor cell lines did not express Rb protein and either failed to express or showed barely detectable expression of cyclin D1 protein. This correlation supports previous indications of the dependence of cyclin D1 expression upon the production of functional Rb protein and is illustrated by the SV40-transformed cell line, HBL-100, that does not express cyclin D1 owing to the inactivation of Rb by Large T Antigen.

With the exception of one cell line, MDA-MB-157, all of the cell lines that expressed both Rb and cyclin D1 failed to express p16 protein. This contrasts with the cell lines HBL-100 and MDA-MB-231, that lacked both Rb and cyclin D1 expression, but expressed p16 protein. These data suggest that the combined loss of p16 and over-expression of cyclin D1 protein's respectively is a mechanism frequently employed by breast cancer cells to overcome the Rb-mediated G1/S block. It therefore follows that in those tumors lacking Rb protein, the need for cyclin D1 function to advance cells through G1 into S-phase is obviated (as in the cell lines DU4475, COLO 591 and MDA-MB-231). Three of the pRb-expressing cell lines, T47-D, MDA-MB-157 and Hs578T, demonstrated only moderate levels of cyclin D1 (approximately equivalent to MCF-12A). This would suggest that the absence of p16 expression (without the accompanying over-expression of cyclin D1) may be sufficient to overcome Rb activity in a sub-group of breast tumors. It should be noted that DU4475 and COLO-591 are unusual in that they fail to express both Rb and p16 proteins. These data confirm previous observations of a high frequency of p16 inactivation in breast cancer (Geradts and Wilson, 1996; Xu et al., 1994; Brenner and Aldaz., 1994).

Western analysis of four pairs of normal and tumor breast tissues lent further support to the cell line data by also demonstrating a positive correlation between Rb and cyclin D1 protein expression (Figure 2). Three of the four tumor extracts expressed Rb protein and showed significant over-expression of cyclin D1 protein relative to the corresponding normal tissue controls. Furthermore, the fourth tumor, COBRC4(T), expresses only barely detectable levels of both Rb and cyclin D1 proteins. We then addressed the question of whether the high level of cyclin D1 protein expressed by tumor's COBRC1(T), 2(T) and 3(T) was an event involved in tumorigenesis or merely a reflection of their greater proliferative rate compared to the corresponding normal tissues. Thus, the western blots previously probed with the cyclin D1 antibody were stripped and reprobed with anti-human cdc2 (PSTAIRE) antibody (Figure 2). Interestingly, the tumor COBRC4 (T) which expressed only barely detectable levels of cyclin D1 protein, showed a high level of PSTAIRE expression whereas two of the remaining three tumors that over-expressed cyclin D1, did not express elevated levels of PSTAIRE protein. The fourth tumor, COBRC2 (T), shows elevated levels of both cyclin D1 and PSTAIRE proteins. This data indicates that the expression of cyclin D1 in the tumor's is not a function of the cellular proliferation rate in the tumor cells but is etiologic in breast tumorigenesis.

The p16 protein was not detected in any of the normal nor tumor tissue samples assessed by western blot analysis. This was not too surprising since Geradts et al. (1996) recently demonstrated that p16 protein expression is more difficult to detect than Rb protein expression in a range of normal tissue types, particularly in breast tissue. It is therefore likely that the western blot procedure is insufficiently sensitive for the detection of p16 protein expression in tissue extracts.

Western blotting analysis of protein expression in breast tumor tissue extracts is limited by the fact that tumors are, to varying degrees, infiltrated with normal cells. We are therefore in the process of optimizing conditions for the immunohistochemical detection of cyclin D1, Rb and p16 protein expression in breast tumor tissue sections. In this manner we will be able to demonstrate with greater accuracy the differences in protein expression between normal and tumor cells within a particular tissue section. This may be especially

important in establishing correlations between the expression of the breast cancer tumor suppressor genes, BRCA1 and BRCA2 and cell cycle regulatory defects (see below). This work is being performed in collaboration with Dr. Wilbur Franklin (Department of Pathology, University of Colorado Health Sciences Center).

Mechanism's of cyclin D1 protein over-expression in breast cancer.

Amplification and elevated transcription of the cyclin D1 gene

Using a 1.1 kb probe from the 5' end of the cyclin D1 cDNA, the panel of breast cancer cell lines were assessed for the copy number of the gene. The MCF-12A cell line was shown to contain two normal copies of chromosome 11 by karyotype analysis (Dr. Leila Garcia, personal communication) and thus was a suitable diploid control for the cyclin D1 locus at 11q13. The cyclin D1-specific hybridization signals in the tumor cell lines were compared directly to that of MCF-12A to determine the copy number of gene. Visual inspection indicated that all 9 of the cell lines that expressed cyclin D1 protein showed amplification of the gene. Densitometric analysis of the cyclin D1 hybridization signals normalized to the B-actin diploid locus, indicated 2-fold amplification in MDA-MB-330 and MDA-MB-231, 3-fold amplification in MCF-7, MDA-MB-175VII and MDA-MB-361, 5-fold amplification in ZR75.1 and 10-fold amplification in MDA-MB-415. Overall, there was a moderate correlation between amplification and protein expression and in particular, MDA-MB-231 showed amplification of the cyclin D1 gene but did not show detectable expression of the protein.

In contrast, analysis of cyclin D1 transcription identified a better correlation between mRNA and protein expression in the cell lines. By comparison to the MCF-12A transcript level and normalization to B-actin transcript levels, densitometric analysis showed 2-fold cyclin D1 mRNA expression in MCF-7, MDA-MB-330, ZR75.1, MDA-MB-175VII, MDA-MB-361 and 4-5-fold expression in MDA-MB-415. The data from both the Southern and northern analysis indicate that the mechanism of over-expression of the cyclin D1 protein involves both DNA amplification and increased transcription of the gene. However, whereas the RNA and protein expression patterns show an good correlation, the degree of DNA amplification is not directly proportional to the protein expression. Indeed, it may occur in the absence of gene expression as in the case of MDA-MB-231.

Post-translational stabilization of the cyclin D1 protein

Stabilization of the cyclin D1 protein was assessed by determination of it's half-life in the cell lines that expressed detectable levels of the protein by western blot analysis. Sub-confluent, cultures of each of the lines were treated with cycloheximide and harvested at given intervals thereafter . With the exception of ZR75.1, all of the cell lines assessed demonstrated the expected cyclin D1 half-life of approximately 20-30 minutes (Figure 3). Cyclin D1 protein levels remained constant in the ZR75.1 cells for at least the first 90 minutes with approximately 60% of the protein remaining at 150 minutes post cycloheximide treatment. Since only one of the nine breast cancer cell lines assessed, showed an increase in he half-life of cyclin D1 protein, it is possible that the post-translational stabilization of cyclin D1 protein may be employed by a small proportion of breast tumors as a further mechanism for maintaining elevated levels of cyclin D1 protein.

Homozygous deletion of the p16 gene is a frequent mechanism of inactivation in breast cancer.

Since ten of the twelve tumor cell lines did not express p16 protein, we assayed the cell lines for the homozygous deletion of the p16 locus at chromosome 9p21. The cell lines MDA-MB-231, MDA-MB-157 and HBL-100 that expressed p16 were included as positive controls for amplification. This was achieved by subjecting the cell line DNA to multiplex PCR analysis using oligonucleotide primers designed from intron 2 of the p16 gene in combination with primers from the D9S199 locus. Since the D9S199 locus is localized distal to the p16 gene at 9p23 and therefore unlikely to be within the homozygously deleted region on chromosome 9p, it was employed as a positive control for amplification. The two normal breast epithelial cell lines, MCF-12A and HBL-100, and six of the ten tumor cell lines (that did not express p16 protein) successfully amplified both the p16 and D9S199 amplicons. However four tumor cell lines showed D9S199 amplification but failed to amplify the p16 product (figure 4). Thus, the homozygous loss of the p16 locus accounted for the lack of p16 protein expression in 40% of the cell lines studies. The remaining 60% of cases may therefore result from mechanisms including structural modifications of the gene such as mutations or deletions; or alterations in the transcription of the gene induced for example, by methylation. Experiments to determine whether or not methylation of the p16 gene occurs in those cell lines that fail to express p16 protein, but are not homozygously deleted for the gene, are in progress using a PCR-based assay (Herman et al., 1996).

One of the cell lines, MDA-MB-157, was exceptional in that it expressed both Rb and p16 proteins. Since mechanisms of p16 inactivation result in a lack of protein expression, we propose that the p16 protein expressed in this cell line is active but inhibited from exerting its regulatory function. Recently Wolfel et al. (1995) reported mutations within cdk4 that prevented binding of p16. This resulted in constitutive cyclin D1/cdk4 kinase activity. It is possible that MDA-MB-157 may harbor such a mutation that inhibits cdk4/p16 binding and thus results in a functionally p16-negative cell line. We plan to test this hypothesis by determining both the components and kinase activity of cdk4 complexes in MDA-MB-157 followed, if appropriate, by mutational analysis.

Construction of antisense cyclin D1 and sense p16 adenoviral vectors.

As discussed earlier all but one of the Rb-expressing breast cancer cell lines demonstrates concomitant cyclin D1 expression and a lack of p16 protein expression indicating the role of the constitutively active cyclin D1/Cdk4 complexes in overcoming the Rb-mediated barrier to S-phase. Three groups have examined the effects of correcting either the abnormal cyclin D1 over-expression or lack of p16 expression in human and mouse malignancies. Following transfection with antisense cyclin D1 both a human esophageal cell line and a mouse lung cancer cell line were suppressed in their ability to induce tumor formation in nude mice (Zhou et al., 1995; Schrump et al., 1995). Jin et al. (1995) have shown that the introduction of p16 into non-small cell lung cancer cell lines lacking p16 expression using a recombinant adenoviral vector greatly reduces their ability to induce tumors in nude mice. These data indicate that the over-expression of cyclin D1 and/or inactivation of p16 do indeed play a role in the development of human malignancy and that the correction of just one of these abnormalities is sufficient to induce reversion to the non-transformed state.

We initially proposed to clone antisense cyclin D1 into the pRc/RSV and pRc/CMV mammalian expression vectors (Invitrogen). However, owing to recent studies that have demonstrated highly efficient transformation of mammalian cell lines using adenovirus-based vectors compared to both retroviral and plasmid vectors (Badie et al., 1994; Liu et al., 1995; Qian et al., 1995), we choose instead to use the recombinant adenoviral vector, 327$_{Bst}$ B-gal (gift from Dr. Jerome Schaack, Department of Microbiology, University of

Colorado Health Sciences Center), for the introduction of antisense cyclin D1 and sense p16 into breast cancer cell lines. Candidate breast cancer cell lines for these experiments were chosen based upon the fact that they demonstrated (a) over-expression of cyclin D1 protein and a lack of p16 protein expression; and (b) tumorigenicity in nude mice. As a control, a breast cancer cell line was also chosen that lacked cyclin D1 protein but expressed p16, and was also tumorigenic in nude mice.

Assessment of breast cancer cell line tumorigenicity

Six of the twelve breast cancer cell lines in the panel showed over-expression of cyclin D1 protein but the majority of these were previously shown to be non-tumorigenic in nude mice (Cailleau et al., 1974; Hackett et al., 1977; Cailleau et al., 1980). However, there was contradictory information and a lack of information regarding the tumorigenicity of MCF-7 and ZR75.1 respectively both of which showed over-expression of cyclin D1 protein and lacked p16 expression. MDA-MB-231 which lacked cyclin D1 but expressed p16 protein was previously shown to be tumorigenic in nude mice (Price et al., 1990). To determine the tumorigenicity of MCF-7 and ZR75.1 and confirm the tumorigenicity of MDA-MB-231, each cell line was injected at 5×10^6 cells subcutaneously into the right shoulder of four male athymic nude mice. Tumor dimensions were measured bi-weekly for 28 days at which time the mice were euthanized and the tumors resected and weighed. None of the four mice injected with MCF-7 cells developed tumors indicating that this cell line was not a suitable candidate for transfection. However, all four of the mice injected either with the ZR75.1 or MDA-MB-231 cell lines developed tumors at the injection site averaging a weight of 0.15 g for ZR75.1 and 0.6 g for MDA-MB-231. Based upon these findings we choose ZR75.1 as the test cell line and MDA-MB-231 as the negative control cell line for the introduction of (a) the adenoviral-antisense cyclin D1 construct, and (b) the adenoviral-sense p16 constructs.

Characteristics of the Adenoviral vector 327_{Bst}B-gal

The vector 327_{Bst}B-gal is a recombinant adenovirus serotype 5. The vector lacks the E1 region (containing the E1a and E1b genes) which is essential for replication, and contains a partially disrupted E3 region the latter of which appears to be involved in the host cell immune response (Schaak et al., 1995). The E1 region has been replaced by the E. coli B-Galactosidase gene driven by the CMV immediate early promoter, and a unique BstBI site has been inserted 3' to the B-Galactosidase gene. As a result the vector is only capable of replicating in the Ad5-transformed cell line 293, a human kidney, embryonal cell line that expresses both E1a and E1b. To construct a recombinant adenovirus expressing a given gene, the latter is first sub-cloned into a plasmid vector PACCMVpLpA (gift from Dr. Jerome Schaack, Department of Microbiology, University of Colorado Health Sciences Center) under the control of a CMV promoter. The plasmid contains sequences homologous to the left' end of Ad5 such that following co-transfection of linearised PACCMVpLpA and 327_{Bst}B-gal digested with BstBI into 293 cells, overlap recombination will occur between the plasmid and viral sequences. The resulting recombinants will appear as clear plaques upon X-gal staining (which will then be isolated) whereas religation of the viral arms will result in blue plaques.

Determination of the efficiency of 327_{Bst}B-gal infection of ZR75.1 cells

Following the calculation of the viral titer at 10^8 pfu/ml and isolation of 327_{Bst}B-gal DNA, we determined the concentration of virus necessary to infect the ZR75.1 cells at a high efficiency without accompanying cytopathic effects upon the cells. ZR75.1 cells were infected with 10^5, 10^3 and 10 pfu/ml of the viral stock and after 24 and 48 hours respectively, the cells were stained with X-gal to determine the proportion expressing B-

Galactosidase (as judged by the development of a blue coloration). After 24 hours approximately 70-80% of the cells infected with 10^5 pfu/ml and 50-60% infected with 10^3 pfu/ml were blue (Figure 5). By 48 hours post-infection, 100% of the cells infected with 10^5 pfu/ml and approximately 80% infected with 10^3 pfu/ml were blue. Further, the proportion of blue cells in the plates infected with 10 pfu/ml was approximately 30%. However, there was an associated increase in the cytopathic effect after 48 hours in the cells infected with 10^3 and 10^5 pfu/ml viral stock. We therefore concluded that the optimal concentration for infection of ZR75.1 cells with the recombinant adenovirus was between 10^3 and 10^4 pfu/ml followed by removal of the virus-containing media after 18 hours.

Immunogenicity of 327_{Bst}B-gal in nude mice

Previous reports have indicated the immunogenic effect of adenoviral vectors upon mammalian hosts (Schaack et al., 1995). We therefore assessed the effect of 327_{Bst}B-gal-infected ZR75.1 cells upon nude mice in order to ensure that future tumorigenicity assays would not be compromised by a host inflammatory response. Although the adenoviral E3 gene had been partially disrupted in this vector, it was not known if the remaining gene product was sufficient to elicit an immune response. ZR75.1 cells were infected with 10^4, 10^6 and 10^7 pfu/ml viral stock (concentrations that were either equal to or in excess of the amounts that we proposed to use for the antisense cyclin D1 and sense p16 experiments) and at 24 hours post-infection, harvested and injected in duplicate at 5 x 10^6 cells subcutaneously into male athymic nude mice. As a control two mice were also injected with non-infected ZR75.1 cells. The mice were monitored bi-weekly for signs of inflammation at the injection site and for tumor development. After 28 days, the mice were euthanised, examined both externally and internally at the injection site and the tumors resected. None of the mice showed evidence of inflammation. Furthermore, the sizes of the tumors that grew in the mice injected with or without 327_{Bst}B-gal-infected ZR75.1 cells were equivalent indicating that the viral vector itself does not suppress the tumorigenicity of the ZR75.1 cells.

Construction of a PACCMVpLpA-antisense cyclin D1 vector

A 1.1 kb HindIII-XbaI fragment from the 5' end of the cyclin D1 cDNA (Invitrogen) was subcloned in the antisense orientation into the XbaI-HindIII site of PACCMVpLpA. Initially, the ligations were transformed into DH5α cells. However, the resulting clones were all highly rearranged indicating a high degree of recombination in this particular bacterial host. We therefore choose to use the rec BC⁻ strain, SURE (Stratagene), from which we obtained stable PACCMVpLpA-antisense cyclin D1 transformants.

Co-transfection of PACCMVpLpA-antisense cyclin D1 constructs with 327_{Bst}B-gal adenoviral vector DNA into 293 cells

Five micrograms of each of the two stable PACCMVpLpA-antisense cyclin D1 constructs were linearised with ClaI and two micrograms of the CsCl-purified 327_{Bst}B-gal DNA were digested with BstBI to cut 3' to the B-Galactosidase gene. We then individually co-transfected 5 micrograms of each of the plasmid construct DNA digests with one microgram of the 327_{Bst}B-gal vector DNA into 293 cells by calcium phophate precipitation. Clear plaques representing overlap recombinants between 327_{Bst}B-gal and each of the PACCMVpLpA-antisense cyclin D1constructs will be isolated and subjected to restriction enyme analysis to identify stable recombinants containing the antisense cyclin D1 gene. Based upon our earlier experiments to determine infection efficiency, ZR75.1 and MDA-MB-231 cells will then be infected with 10^3 and 10^4 pfu/ml recombinant adenoviral-antisense cyclin D1 constructs. The resulting transfectants will be assessed in vitro for

growth rate and anchorage-independent growth, and in vivo for tumorigenicity in nude mice.

<u>Construction of a PACCMVpLpA-sense p16 constructs</u>

We subcloned a functional 0.5 Kb BamHI-XbaI fragment from the 5' end of the p16 gene (gift from Dr. A. Kamb, Myriad Genetics, Utah) into PACCMVpLpA. We plan to co-transfect PACCMVpLpA-sense p16 constructs and 327_{Bst}B-gal DNA into 293 cells as described above to isolate recombinant adenoviral-p16 constructs. The latter will then be transfected into the ZR75.1 and MDA-MB-231 cell lines to determine if the reintroduction of p16 into ZR75.1 is sufficient to cause reversion to the non-transformed phenotype as judged by in vitro and in vivo assays.

Allelic loss analysis of breast cancer cell lines and tumors at the BRCA1 and BRCA2 loci

Mutations at the BRCA1 locus on chromosome 17q21 account for approximately 40-60% of familial early onset breast cancer and approximately 80% of familial breast/ovarian cancer syndrome. Mutations at the BRCA2 locus on chromosome 13q12-13 account for 40-50% of familial breast cancers but are more frequently detected in pedigrees including male breast cancer sufferers and sporadic breast tumors (Wooster et al., 1995). The functions of the proteins encoded by the BRCA1 and BRCA2 tumor suppressor genes have yet to be elucidated. Indeed, there is still some controversy as to the sub-cellular distribution of the BRCA1 and BRCA2 proteins in normal and tumor breast epithelial cells (for a review, see Hoffman., 1996).

The panel of breast cancer cell lines were genotyped at the BRCA1 and BRCA2 loci and the frequencies of heterozygosity obtained compared with the reported frequency of heterozygosity in the normal population. The latter provided an indication as to the proportion of cell lines that had undergone loss of an allele and were therefore likely to contain a mutation in the remaining copy of the gene. We then assessed the panel for a correlation between the BRCA1 and BRCA2 genotypes of the cell lines and the expression patterns of particular cell cycle regulatory proteins (as shown in Figure 1). DNA was prepared from the panel of normal and tumor breast cancer cell lines and subjected to PCR analysis at the BRCA1 (D17S855) and BRCA2 (D13S267) tumor suppressor loci. The primers flank highly polymorphic microsatellite dinucleotide (CA)n repeats (Research Genetics Inc.). One of the primers from each pair was end-labelled with [γ32P]-ATP, and the PCR products electrophoresed on a 32% formamide/7% acrylamide gel. Only 58% and 44% of the cell lines retained heterozygosity at the D17S855 and D13S267 loci respectively. Since the reported frequencies of heterozygosity at these loci in the normal population are 82% and 75% respectively, it is likely that many of these cell lines have undergone loss of the wild-type allele, thus revealing the recessive mutant allele.

Interestingly, in two of the cell lines that do not express Rb nor cyclin D1 protein (DU4475 and MDA-MB-231), both BRCA1 and BRCA2 loci are heterozygous, and thus highly likely to be functionally active. The cell line, COLO 591 that also lacks both Rb and cyclin D1 protein expression was heterozygous at the BRCA2 locus only. Only two of the nine cell lines that expressed both Rb and cyclin D1 proteins showed evidence of heterozygosity, in both cases at the BRCA1 locus. These preliminary data suggest that the initial loss of Rb protein expression in breast epithelial cells and consequent lack of a G1/S block is sufficient for transformation to breast cancer without the need for further functional inactivation of the tumor suppressor genes BRCA1 and/or BRCA2.

The studies of breast cancer cell lines are limited by the absence of a corresponding normal cell line derived from the same patient. It is therefore not possible to assume that a homozygous genotype necessarily indicates loss of an allele. To overcome the problems

associated with the analysis of unmatched tumor cell lines lacking a normal counterpart, we have undertaken a collaboration with Dr. Wilbur Franklin to analyse matched pairs of tumor and normal breast tissue both for expression of Rb protein by immunohistchemistry and for allelic loss at the BRCA1 and BRCA2 loci. Since breast tumors are often heavily contaminated with normal cells, we have isolated DNA from microdisected normal and tumor cells from 6 tissue sections and optimised the conditions for the PCR amplification of these samples at the BRCA1 and BRCA2 loci. Four of the six pairs of samples were constitutionally heterozygous (ie., the normal DNA was heterozygous) at the BRCA2 locus and of these, two of the tumors had undergone allelic loss whilst two retained heterozygosity. All six of the pairs were constititually heterozygous at the BRCA1 locus and of these, two of the tumors had undergone allelic loss and three had retained heterozygosity. Interestingly, the sixth tumor showed evidence of microsatellite instability in that it shared one allele with the corresponding normal DNA but exhibited a second allele of an altered size relative to the corresponding normal DNA. If our hypothesis regarding the relationship between Rb and BRCA1/BRCA2 expression holds, then we would expect that those tumors that have undergone loss of heterozygosity at the BRCA1/BRCA2 loci to express Rb protein and those that retain heterozygosity to lack Rb expression. We have recently optimised the conditions for the immunohistochemical analysis of the breast tumor tissue sections for Rb expression but as yet do not have sufficient data to confirm the suggestion from the cell line analysis.

We are also in the process of microdisecting normal and tumor cells from another 14 breast tumor sections to increase our sample number to twenty. A larger cohort of matched pairs of tumor and normal breast tissues will enable us to determine the relationship between expression of cell cycle regulatory proteins such as Rb and the breast cancer tumor suppressor genes BRCA1 and BRCA2.

Conclusions:

Our studies, along with studies from other laboratories, have indicated that defects of one type or another in the cyclin D1-Rb-p16 cell cycle regulatory system are present in the vast majority of breast cancer cells. In the case of cyclin D1, defects in expression are associated with increased amplification of the cyclin D1 gene, as well as, in one case, a change in the half-life of the protein, but are most closely correlated with the level of cyclin D1 mRNA expression. The use of the adenoviral vectors encoding sense and antisense genes for cell cycle regulatory proteins that we are developing should allow us to directly demonstrate the contribution of defects in the expression of these proteins to the malignant state in breast cancer, and possibly provide targets for gene therapy strategies that may be developed in the future.

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Table 1 Expression of cyclin D1, Rb and p16 proteins in breast cancer cell lines and tissues

Cell line/ tissue name	Histology	Rb protein	Cyclin D1 protein	p16 protein
Non-tumor cell lines				
MCF-12A	Normal epithelial	+	++	-
HBL-100	Normal epithelial	-*	-	+
Tumor cell lines				
MCF-7	Adenocarcinoma	+	++++	-
MDA-MB-330	Carcinoma	+	++++	-
T47-D	Ductal carcinoma	+	++	-
ZR75.1	Carcinoma	+	++++	-
MDA-MB-157	Medulla carcinoma	+	++	+
MDA-MB-175VII	Ductal carcinoma	+	+++	-
MDA-MB-361	Adenocarcinoma	+	+++	-
MDA-MB-415	Adenocarcinoma	+	++++	-
Hs578T	Ductal carcinoma	+	++/+	-
DU4475	Carcinoma	-/+	-/+	-
COLO 591	not known	-	-	-
MDA-MB-231	Adenocarcinoma	-	-	+
Tissues				
COBRC1 (N)	Normal epithelial	+	+	-
COBRC1 (T)	Ductal carcinoma	+	+++	-
COBRC2 (N)	Normal epithelial	+	+	-
COBRC2 (T)	Ductal carcinoma	+	++++	-
COBRC3 (N)	Normal epithelial	+	+	-
COBRC3 (T)	Carcinoma	+	+++	-
COBRC4 (N)	Normal epithelial	+	-/+	-
COBRC4 (T)	Medulla carcinoma	-/+	-/+	-

The presence or absence of Rb and p16 expression is indicated by a (+) or a (-) respectively. *Although HBL-100 expresses Rb protein the latter is complexed to SV40 T Ag and is therefore non-functional. The levels of cyclin D1 protein (relative to the non-tumor cell line MCF-12A) are indicated as follows: (++++), very high; (+++), high; (++), moderate (equivalent to MCF-12A); (-/+), barely detectable; (-), undetectable. The "COBRC" prefix corresponds to normal (N) and tumor (T) paired patient tissues.
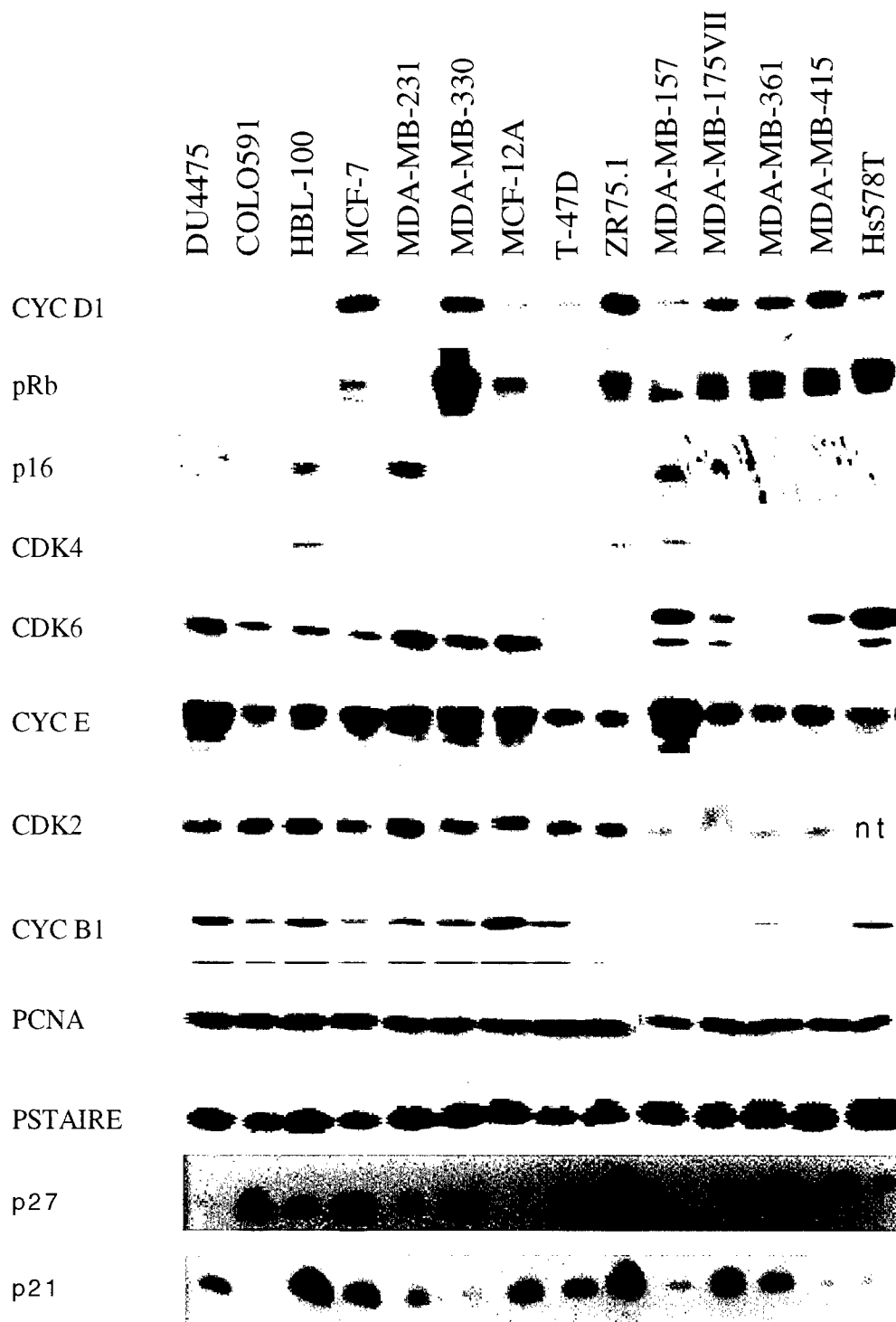
Figure 1 Expression of cell cycle regulatory proteins in normal and tumor breast epithelial cell lines. Cell line extracts were prepared from the indicated cell lines and subjected to SDS/PAGE. Following transfer to Immobilon P membranes they were probed with a panel of 15 antibodies. A representative selection of western blots are shown. The normal breast epithelial cell lines included in the panel were MCF-12A and HBL-100. nt denotes not tested.
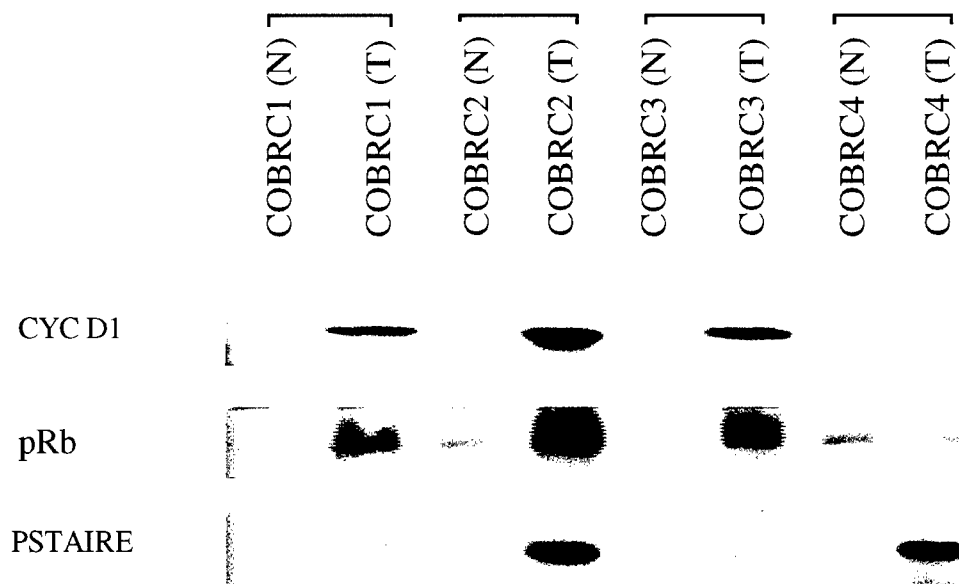
Figure 2 Cyclin D1 and Rb protein expression in matched pairs of normal
and tumor breast tissues. Protein extracts were prepared from breast
tissues, subjected to SDS/PAGE,transferred to Immobilon P membranes,
and probed with antibodies to cyclin D1, Rb and PSTAIRE. Tumor and
normal tissue samples are designated by a "T" and "N" suffix respectively.
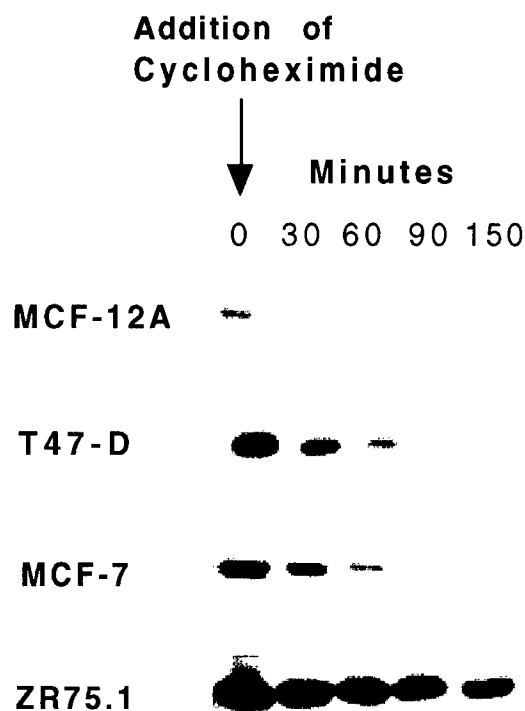
Addition of
Cycloheximide

Minutes

0 30 60 90 150

MCF-12A

T47-D

MCF-7

ZR75.1

Figure 3 The half-life of cyclin D1 protein is extended in the breast cancer cell line, ZR75.1. Log phase cultures of the indicated cell lines were treated with 0.1 mg/ml cycloheximide, harvested at the time intervals shown and extracts prepared. Following SDS/PAGE, the extracts were transferred to Immobilon P membranes and probed with an antibody to cyclin D1.
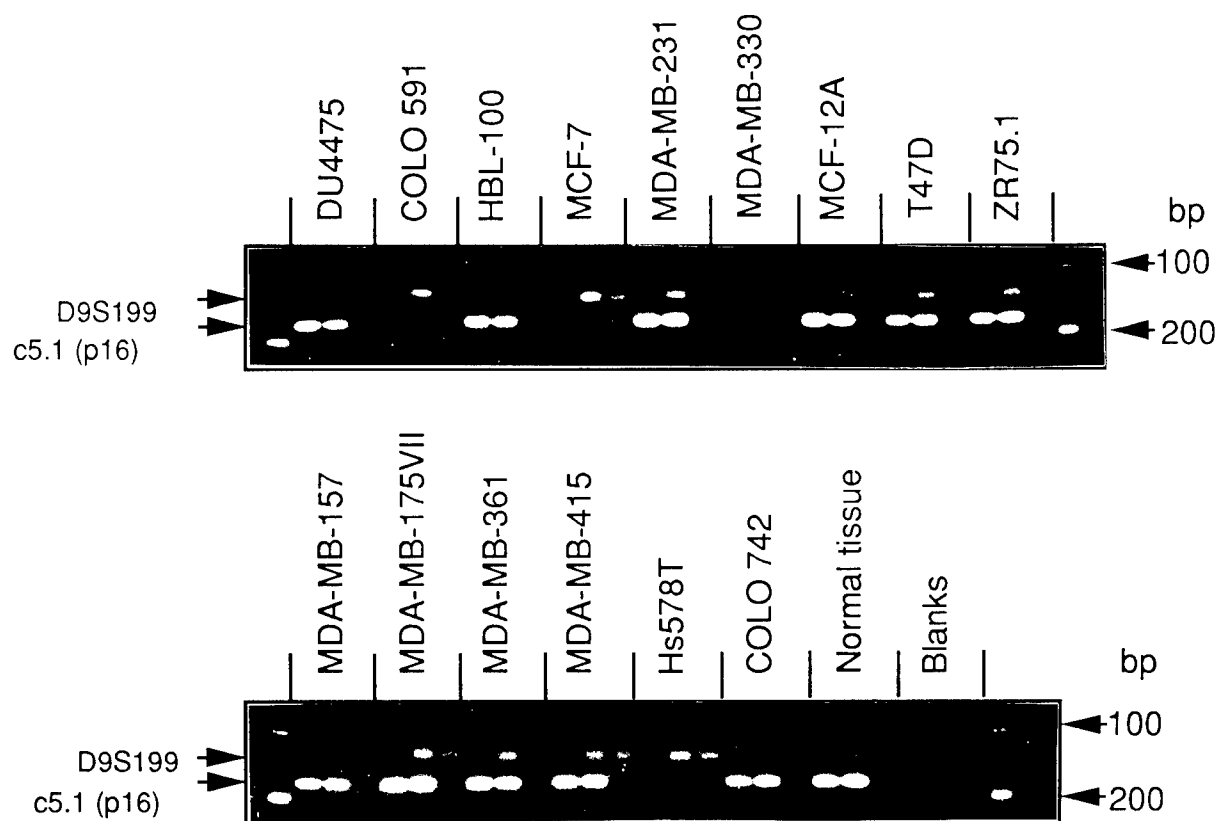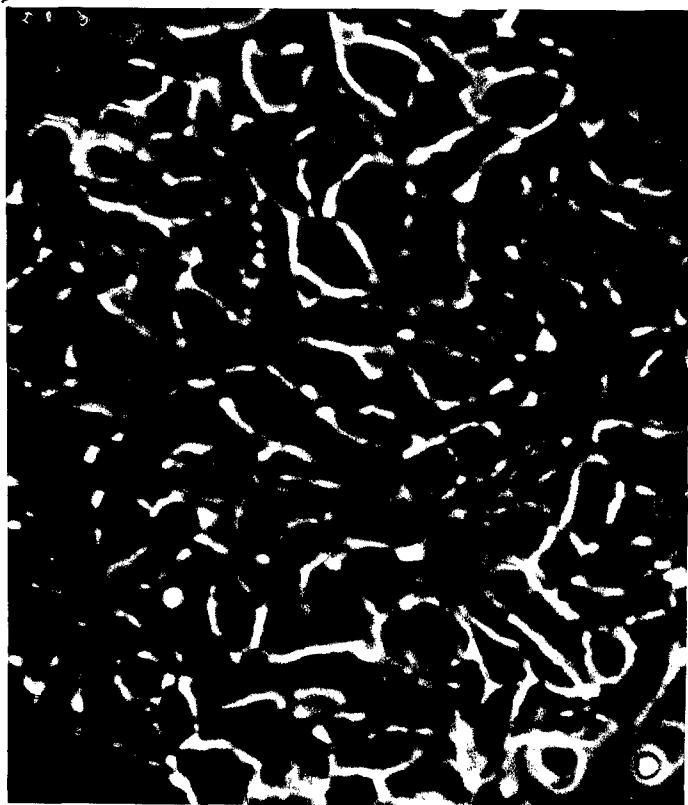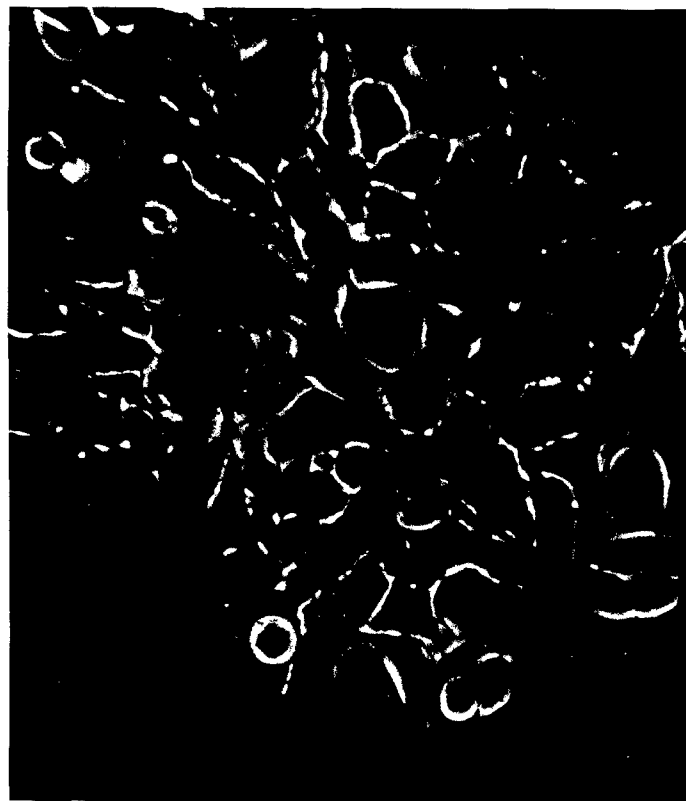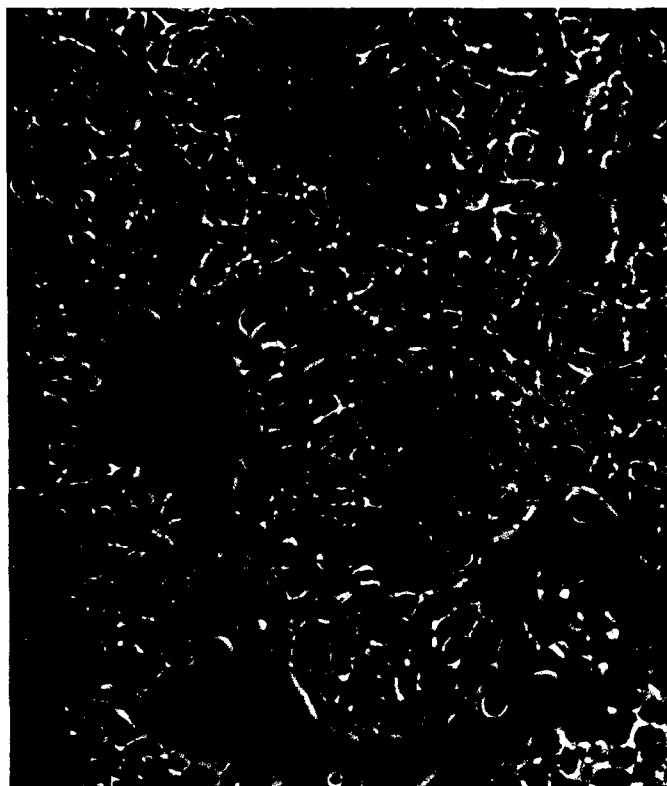
Figure 4 PCR analysis of breast cancer cell lines using oligonucleotide primers to: c5.1 (p16) (first lane under each cell line heading); c5.1 (p16) and D9S199 (second lane under each cell line heading); and D9S199 (third lane under each cell line heading). Following amplification the PCR products were electrophoresed on 3% NuSieve gels. The amplicons were visualized with uv following ethidium bromide staining. Four of the cell lines failed to amplify the c5.1 (p16) product whereas all of the cell lines amplified the control D9S199 product. DNA derived from two normal breast cell lines, MCF-12A and HBL-100, a normal breast tissue sample, and a breast cancer cell line, COLO 742 (not described in the analysis of cell cycle protein expression) were included in the panel. The three lanes under the heading "Blanks" contain all of the components of the PCR reaction mixtures minus DNA to indicate the absence of contamination.

24

A

B

C

Figure 5 Expression of B-galactosidase in 327_{Bst}B-gal-infected ZR75.1 cells.
A), non-infected (20 X mag);
B), infected with 10^5 pfu/ml 327_{Bst}B-gal (20 X mag);
C), infected with 10^3 pfu/ml 327_{Bst}B-gal (10 X mag).
Following infection with 327_{Bst}B-gal, cells were incubated at 37°C for 24 hours, then stained with 1 mg/ml X-gal.

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